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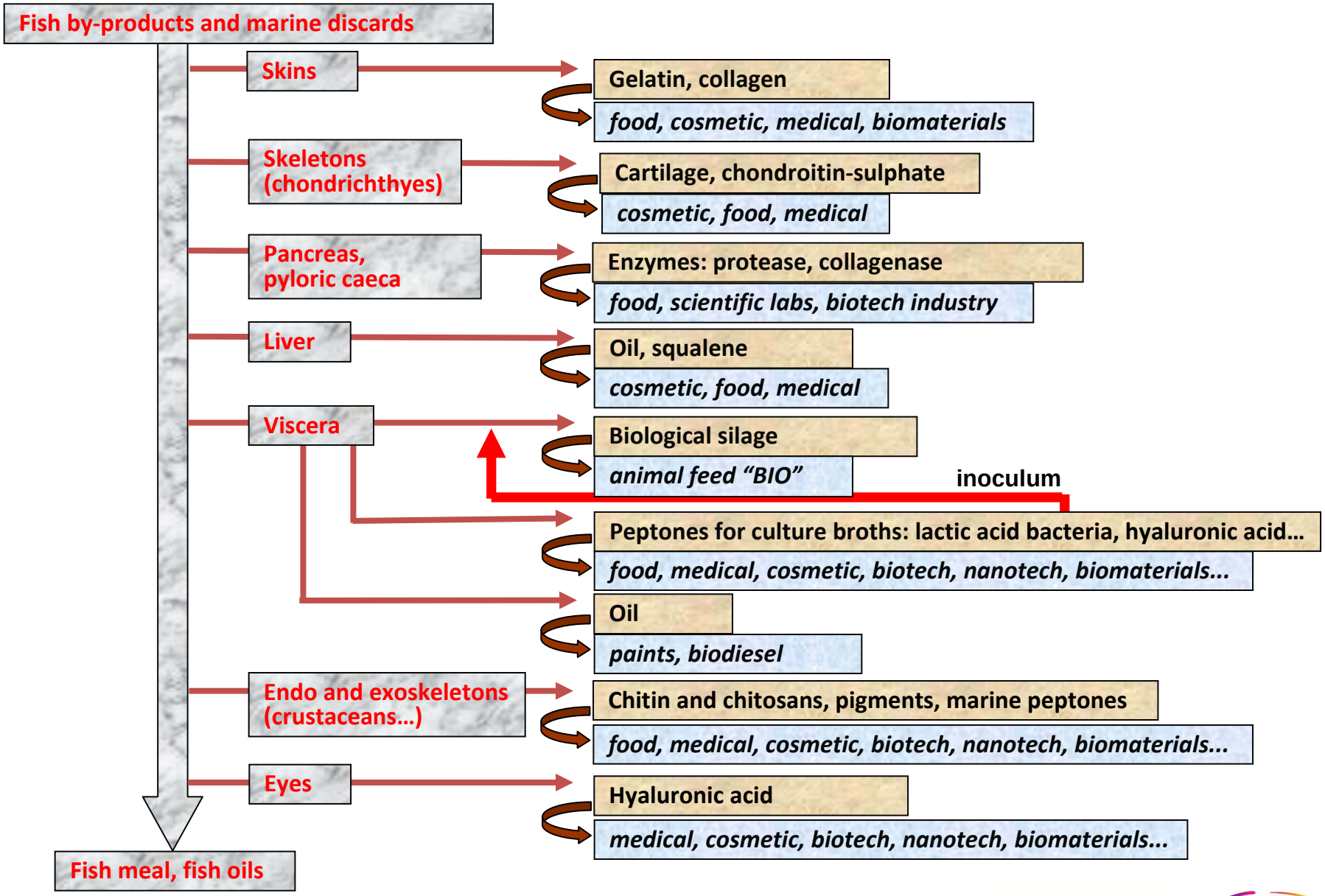
Laboratorio de Bioquímica
Universidade Vigo

Optimization of enzymatic cartilage hydrol is from *Prionace glauca* wastes for the produc on of chondroitin sulphate

José A. Vázquez[1]*, María Blanco[2], Diana Noriega[2,3], Javier Fraguas[1], Lorenzo Pastrana
Carmen G. Sotelo[2] & Ricardo Pérez-Martín[2]

[1]Grupo de Reciclado y Valorización de Materiales Residuales (REVAL), (IIM-CSIC), Vig pain)
[2]Grupo de Bioquímica de Alimentos (BA), (IIM-CSIC), Vigo (Spain)
[3]Departamento de Química Analítica y Alimentaria, Universidad de Vigo, Ourense (Spa
**** jvazquez@iim.csic.es***

Recycling and marine waste valorisation from IIM-CSIC



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STATE OF THE PROBLEM and MOTIVATION

Fishing Port of Vigo

- **Largest fishing port in Europe (fresh and frozen fish).**
- **Largest fishing market in Europe (litoral, big fishes, coastal auctions, etc.).**
- **~1 MTm of fish/year landed, commercialized and moved from the Port of Vigo.**
- **35% are by-products (350000 Tm/year).**

***Prionace glauca* fishery:**

- **50% of sharks landed in Europe were *P. glauca* (>60% in Spain, 2009).**
- **2500 Tm (fresh) were landed in Vigo (2013).**
- **It is the 6th most landed species in Vigo.**
- **33% of life weight is a by-product (visceras, skin and head). 15% of this weight are heads.**

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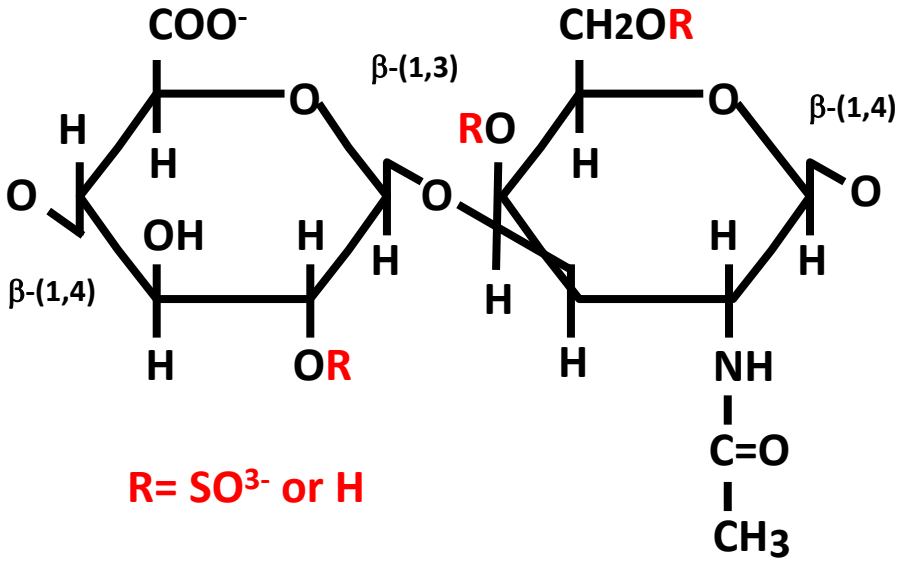
CHONDROITIN SULPHATE from CARTILAGE

Cartilage is a connective tissue formed by chondrocytes cells and an extracellular matrix including proteoglycan and protein fibers (elastin, collagen). Proteoglycan is the covalent association of glycosaminoglycans chains (chondroitin sulfphate, etc.) and proteins (aggrecan, etc.).

Chondroitin sulphate is a linear polysaccharide consisting of repeating disaccharide units of glucuronic acid and sulfated N-acetyl galactosamine, mainly in C4 or/and C6.



R= SO^{3-} or H

Increasing demand in tissue bioengineering, nanomaterials, hydrogels, repair of skin and bone lesions, dental material support, arthritis and osteoarthritis treatment, etc.

OBJECTIVES and WORKING PLAN

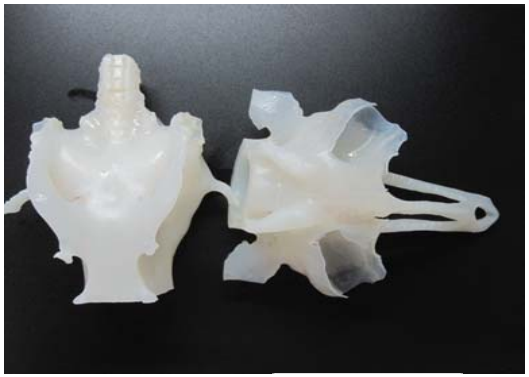
1: To study the first step (enzymatic hydrolysis of cartilage) to extract chondrotin sulphate from shark head wastes.

2: To assess the joint effect of *pH* and *T* in the proteolytic hydrolysis run by alcalase in cartilage of shark head.

3: To optimize the best conditions for the alcalase catalysis by combination of kinetics and response surface methodology approaches.

Cartilaginous material from *P. glauca* head wastes was initially prepared following the next steps:

a) Hot water cooking (80ºC/1 h).
b) Manual or mechanical cleaning of the muscle remains.
c) Homogenization of sample by grinding.



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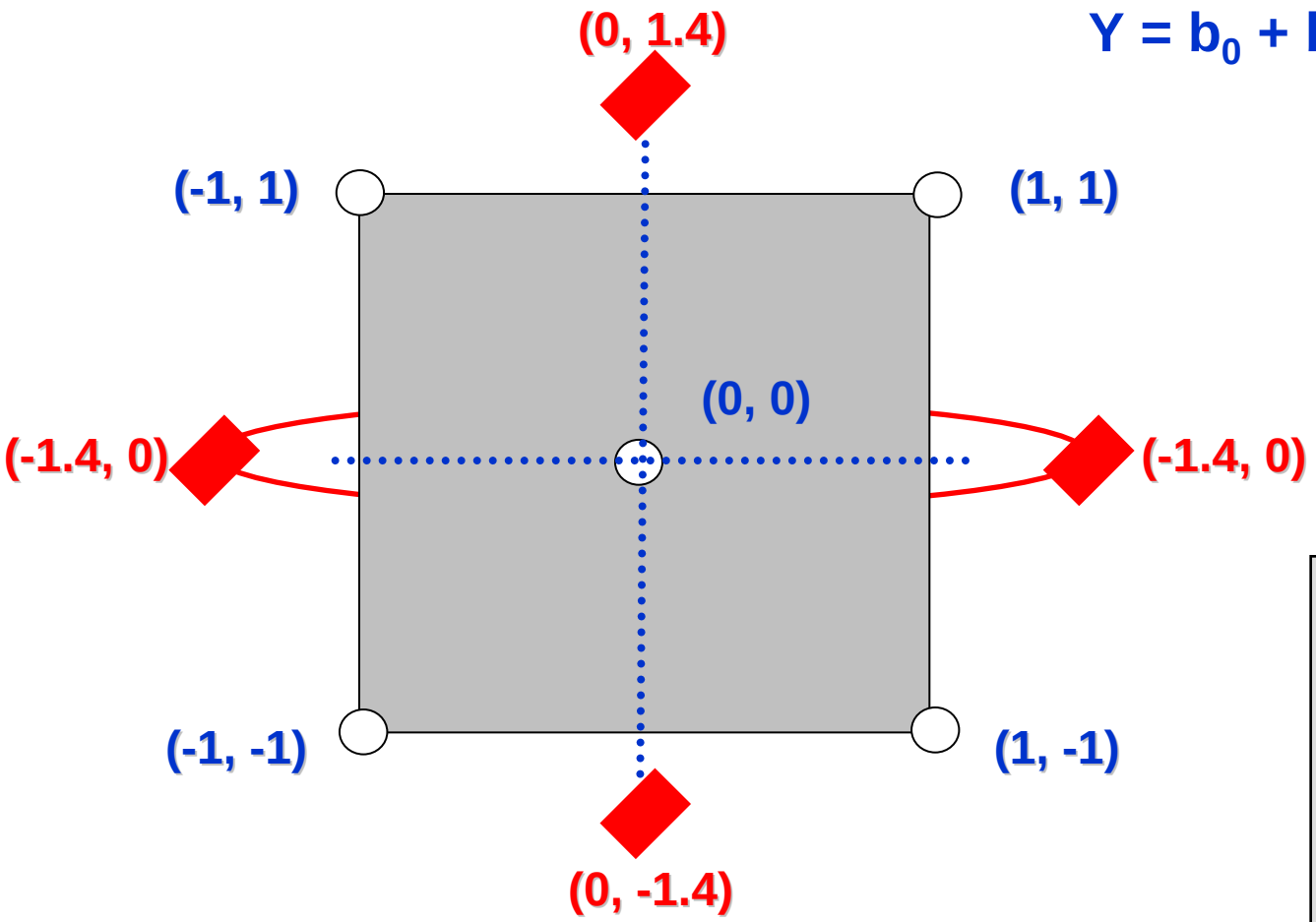
EXPERIMENTAL CONDITIONS (rotatable second order design)



$$Y = b_0 + b_1 pH + b_2 T + b_{12} TpH$$

RESPONSE SURFACE METHODOLOGY (RSM):

1) Parameters significance: t-Student test (α=0.05).
2) Equation consistency: F-Fisher test (α=0.05).
3) Equation-data correlation: R^2_{adj}.

$$Y = b_0 + b_1 pH + b_2 T + b_{12} TpH + b_{11} pH^2 + b_{22} T^2$$

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EXPERIMENTAL CONDITIONS (rotatable second order design)

Nº Exp	T (ºC)	pH	Tcod	pHcod
1	37.3	6.9	-1	-1
2	72.7	6.9	1	-1
3	37.3	11.1	-1	1
4	72.7	11.1	1	1
5	30.0	9.0	-1.41	0
6	80.0	9.0	1.41	0
7	55.0	6.0	0	-1.41
8	55.0	12.0	0	1.41
9	55.0	9.0	0	0
10	55.0	9.0	0	0
11	55.0	9.0	0	0
12	55.0	9.0	0	0
13	55.0	9.0	0	0

Constant conditions:

[alcalase]= 0.3% (v/w), 7.2 AU/kg
R (S:L)= 1:3 (25 g:75 mL H_20)
t= 4 h
Agitation ~ 250 rpm
pH-stat Reactor

CODIFICATION	DECODIFICATION
$V_c = (V_n - V_o)/\Delta V_n$	$V_n = V_o + (\Delta V_n \times V_c)$
V_n: natural value of the variable to codify.	V_0: natural value in the centre of the domain.
V_c: codified value of the variable.	ΔV_c: increment of V_n per unit of V_c

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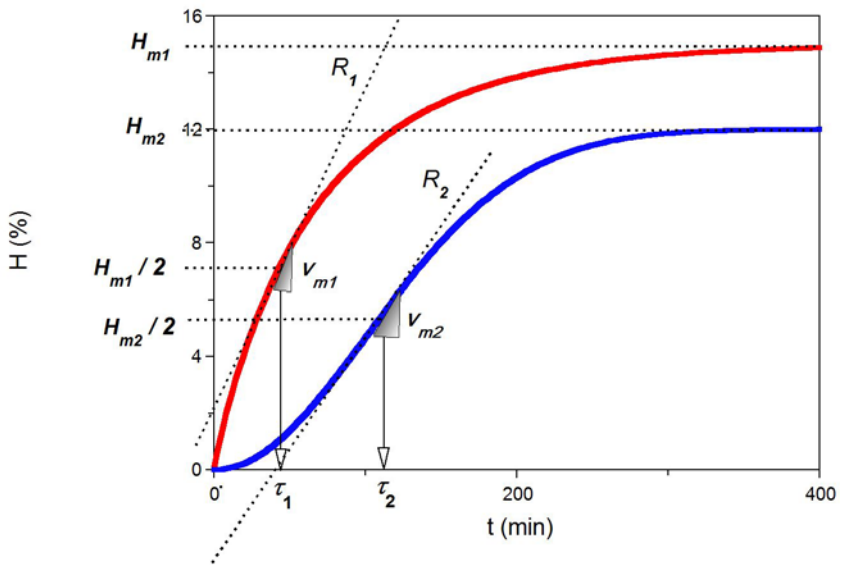
RESULTS OF HYDROLYSIS KINETICS

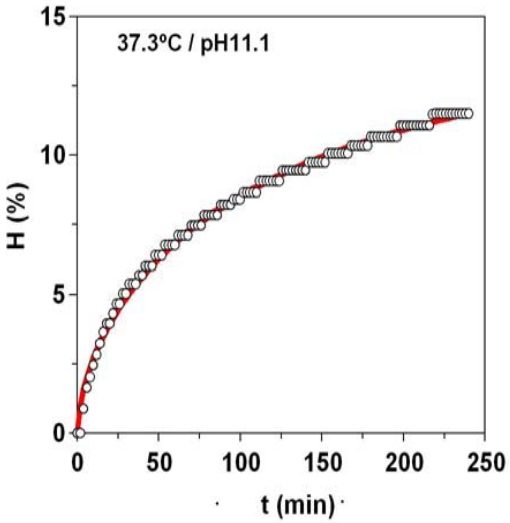


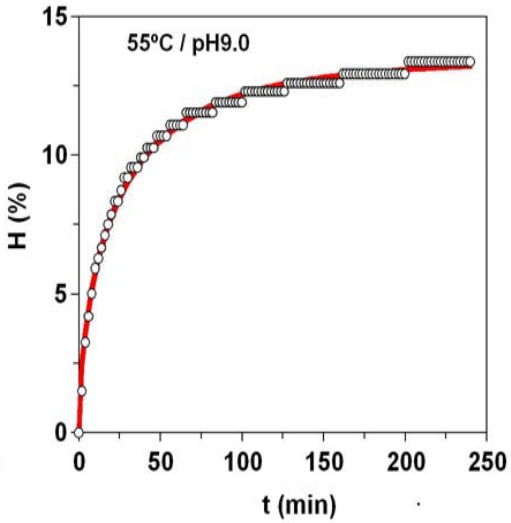
Modelling of kinetics by Weibull equation

$$H = H_m \left\{ 1 - \exp\left[-\ln 2 \left(\frac{t}{\tau} \right)^{\alpha} \right] \right\}$$

$$v_m = \frac{\alpha H_m \ln 2}{2\tau}$$

***H*:** degree of hydrolysis (%).
***t*:** time of hydrolysis (min).
H_m**:** maximum hydrolysis (%).
α**:** parameter of form (dimensionless).
τ**:** time to achieve $H_m/2$ (min).
v_m**:** maximum hydrolysis rate (% min^{-1}).

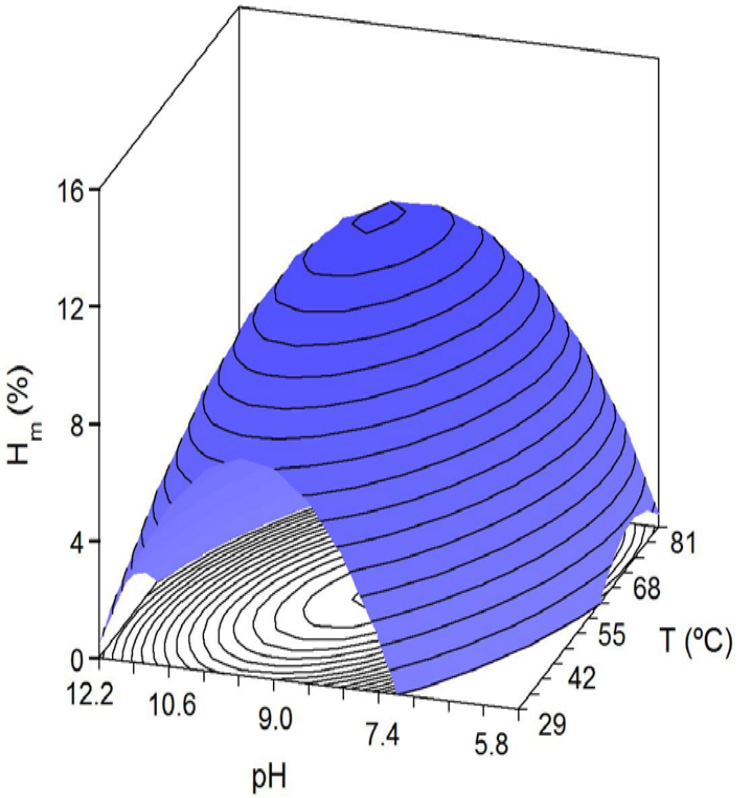




1) It is a flexible equation to model different experimental profiles: sigmoid, hyperbolic, etc.
2) It can be formulated with parameters of enzyme hydrolysis relevance.
3) Perfect agreement among the experimental data and the predicted fittings by equation (R^2>0.991).

ALCALASE HYDROLYSIS OPTIMIZATION

Maximum hydrolysis (H_m) as response (dependent variable)



$$H_m = 13.0 - 3.72TpH - 3.01T^2 - 6.28pH^2$$

$$\frac{\partial H_m}{\partial T} = -3.72pH - 6.02T \qquad -3.72pH_{max} - 6.02T_{max} = 0$$

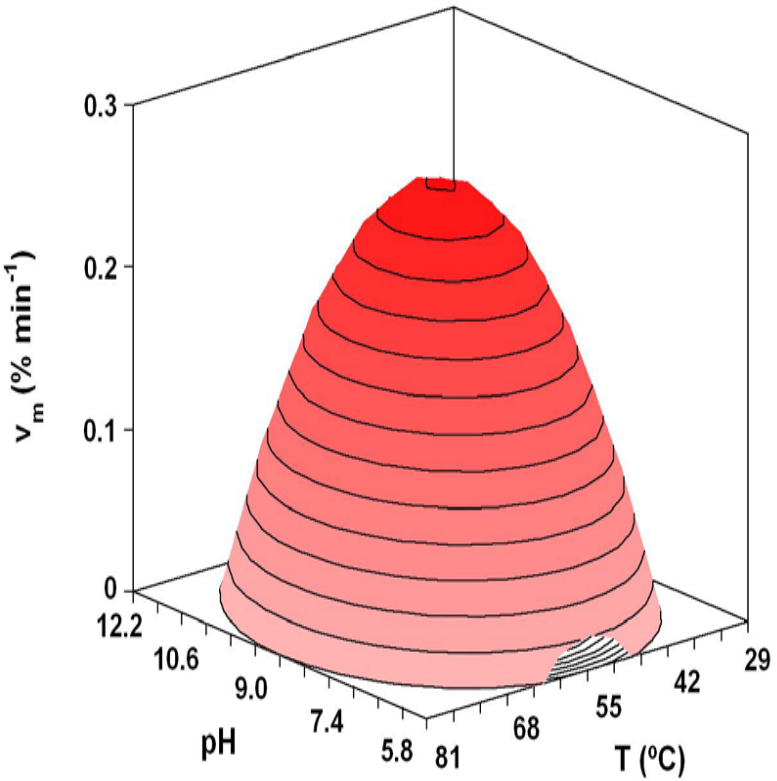
$$\frac{\partial H_m}{\partial pH} = -3.72T - 12.56pH \qquad -3.72T_{max} - 12.56pH_{max} = 0$$

T_{opt}= 55ºC pH_{opt}= 9.0

$R^2_{adj} = 0.720$

ALCALASE HYDROLYSIS OPTIMIZATION

Maximum hydrolysis rate (v_m) as response (dependent variable)



$$v_m = 0.264 - 0.095T^2 - 0.140pH^2$$

$$\frac{\partial v_m}{\partial T} = -0.19T \qquad -0.19T_{max} = 0$$

$$\frac{\partial v_m}{\partial pH} = -0.28T \qquad -0.28pH_{max} = 0$$

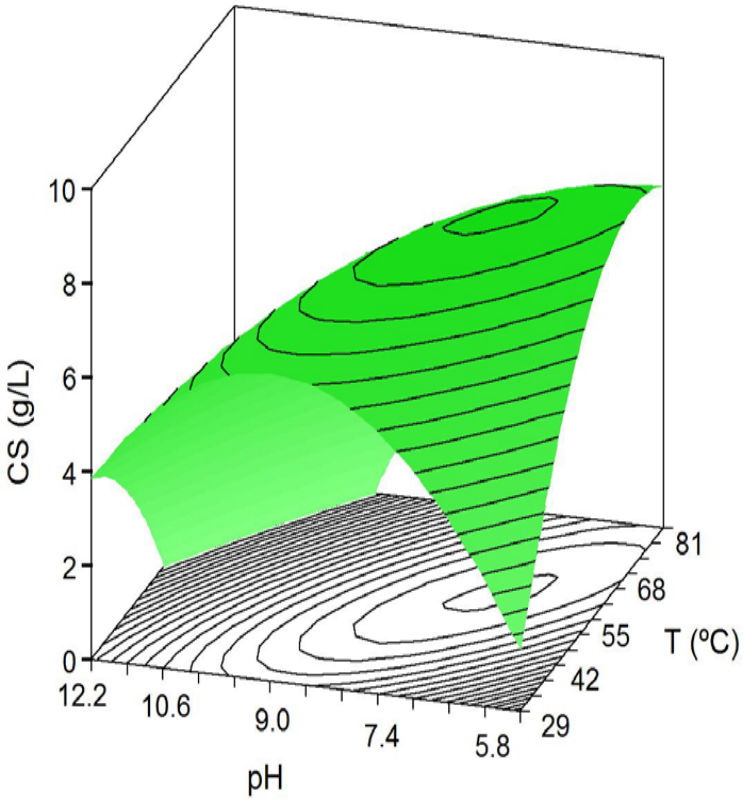
T_{opt}= 55ºC $\qquad$ pH_{opt}= 9.0

$$R^2_{adj} = 0.776$$

ALCALASE HYDROLYSIS OPTIMIZATION

Chondroitin sulphate concentration (*CS*) as response: purified in pseudo-optimal conditions according to *Murado et al. (2009): Biochem. Eng. J. 49 (2010) 126–132*

$$CS = 7.34 - 1.07T - 2.21pH - 2.04TpH - 1.11T^2 - 1.72pH^2$$



$$\frac{\partial CS}{\partial T} = -1.07 - 2.04pH - 2.22T \qquad -1.07 - 2.04pH_m - 2.22T_m = 0$$

$$\frac{\partial CS}{\partial pH} = -2.21 - 2.04T - 3.44pH \qquad -2.21 - 2.04T_m - 3.44pH_m = 0$$

T_{opt}= 59.2ºC pH_{opt}= 7.34

$R^2_{adj} = 0.812$

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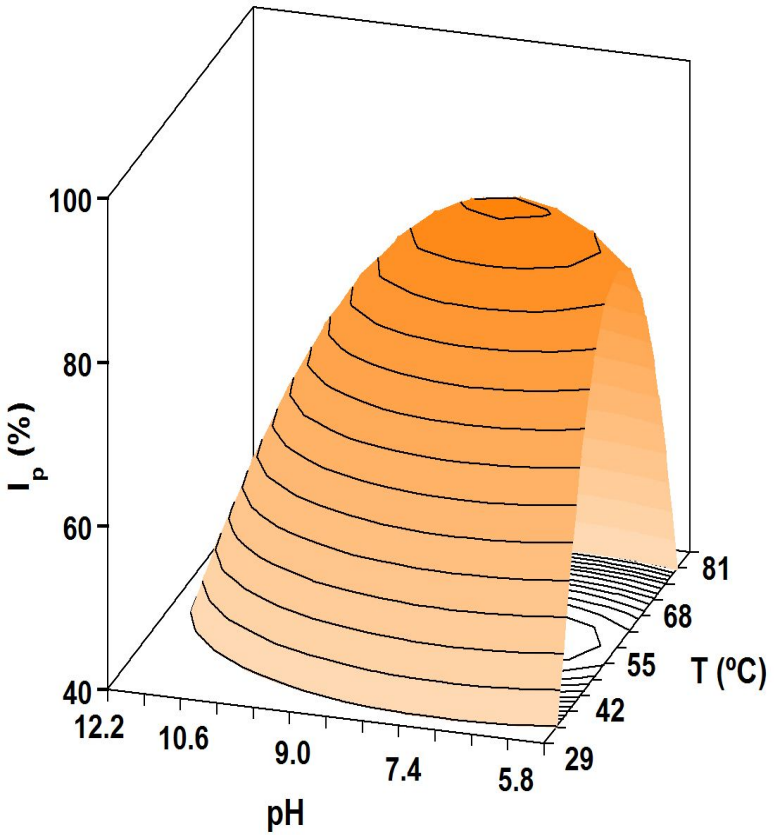
ALCALASE HYDROLYSIS OPTIMIZATION

CS purity in relation to proteins (I_p) as response



$$I_P = 86.87 - 17.38pH - 30.35T^2 - 11.84pH^2$$

$$\frac{\partial I_P}{\partial T} = -60.7T \qquad -60.7T_m = 0$$

$$\frac{\partial I_P}{\partial pH} = -17.38 - 23.68pH \qquad -17.38 - 23.68pH_m = 0$$

T_{opt}= 53.6ºC pH_{opt}= 7.43

$R^2_{adj} = 0.939$

ALCALASE HYDROLYSIS OPTIMIZATION

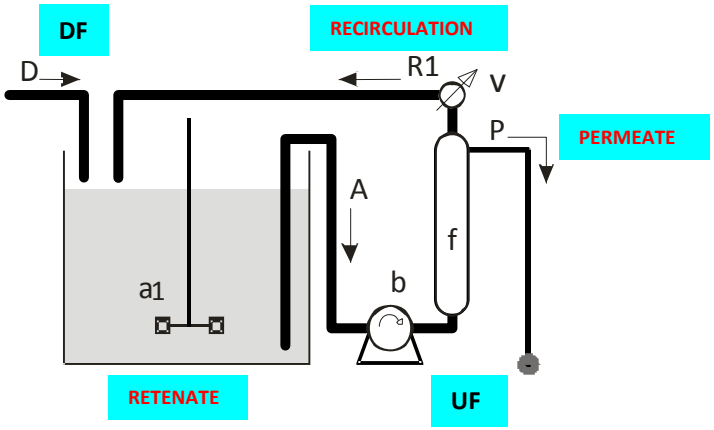
OPTIMAL GLOBAL CONDITIONS (OGc)

RESPONSE (*Y*)	T_{opt} (ºC)	pH_{opt}	Maximum Response (Y_m)
H_m	55.0	9.0	13.0%
v_m	55.0	9.0	0.264 % min^{-1}
CS	59.2	7.34	8.08 g/L
I_P	53.6	7.43	93.05%
OGc	55.7	8.2	12.2% (H_m) 0.244 % min^{-1} (v_m) 7.92 g/L (*CS*) 91.69% (I_p)

FUTURE DEVELOPMENTS

1: To optimize the best conditions for aqueous protein hydrolysis and chondroitin sulphate selective precipitation by alkaline-hydroalcoholic reaction.

2: To purify and maximize the chondroitin sulphate recovery by ultrafiltration and diafiltration performance at different cut-offs (30, 100 kDa…).



3: Physicochemical characterization: molecular weight, type of sulphation and pattern of sulphation.

ACKNOWLEDGEMENTS

This study has been possible thanks to the sponsorship of:

0687_NOVOMAR_1_P: "*Consolidação do Centro Multipolar de Valorização de Recursos Marinhos visando Novos Usos do Mar*".
POCTEP (Programa de Cooperacion Transfronteriza España-Portugal) Programme, INTERREG, EU

BiValBi: "*Biotechnologies to Valorise the regional food Biodiversity in Latin America*".
FP7-PIRSES-GA-2013-611493

INNOSABOR, CDTI. Ministerio de Industria


THANK YOU
FOR YOUR ATTENTION
chondroitin sulphate